Protocol for *Nicotiana tabacum* axenic explants transfection

Materials

Biologic

- *Nicotiana tabacum* axenic explants

Lab Material

- 2 pipettes/ 1 ml
- 1 pipette/ 250 ml
- 1 Erlenmeyer flask/ 1000 mL
- 16 media flasks
- Cotton
- Gauze
- Sclapel, scalpel blades, Kelly tweezers
- Aluminum fold
- Electric grill
- Mosca
- Parafilm
- Eppendorf flask
- Microscope slides
- Cover slip

Equipment

- Laminar flow cabinet
- Autoclave
- Potentiometer
- Analitic scale

Reactives

- Ethylic Alcohol 70%
- Sterile water
- Murashige&Skoog media (MS)
- Hormones at the folowing concentrations: 1.0 mg/L 6-BAP y&0.1 mg/L NAA (Naftalenic acid)
- 5 mg/L de 2,4-D & 0,25 mg/L de Kinetina
- Glyceraldehyde 50% (In distilled water)

The MS culture media

The media will be prepared at the following concentrations: Macronutrients: NH_4NO_3 1650 $mg.L^{-1}$, KNO_3 1900 $mg.L^{-1}$, $CaCl_2.H_2O$ 440 $mg.L^{-1}$, $MgSO_4.2H_2O$ 370 $mg.L^{-1}$, KH_2PO_4 170 $mg.L^{-1}$, $NaH_2PO_4.H_2O$ 85.0 $mg.L^{-1}$. Micronutrients: KI 83.0 $mg.L^{-1}$, H_3BO_3 6.2 $mg.L^{-1}$, $NaMoO_5.2H_2O$ 0.25 $mg.L^{-1}$, $CuSO_4.5H_2O$ 0.25 $mg.L^{-1}$, $CoCl_2.6H_2O$ 0.025 $mg.L^{-1}$, $Na_2EDTA.2H_2O$ 37.2 $mg.L^{-1}$, $FeSO_4.7H_2O$ 27.8 $mg.L^{-1}$. Organic compounds: Nicotinic acid 0.5 $mg.L^{-1}$, Piridoxine.HCl 0.5 $mg.L^{-1}$, Tiamine.HCl 0.1 $mg.L^{-1}$, glycin 2.0 $mg.L^{-1}$), this will be complemented with vegetable growth regulators. For tobacco plant-callus generation, 2.0mg/L of 6-BAP (6-Bencylaminopurine) and 0.1 NAA (Naftalaceticacid), of the already prepared MS media 4.43gr/L will be added with sucrose at a concentration of 30g/L, at this point the pH of the solution will be adjusted at 5.7 with NaOH 1.0N o HCl 1.0N, phytagel to be added in this point (2.5g/L). The media will then be melted in heat and sterilized at the autoclave at 121°C and 15 pounds for 15 minutes, finally the media will be poured at Petri dishes (aprox. 20ml per dish.)

UM5 Media preparation.

MS media will be prepared as specified before and then will be added with 5mg/Ñ of 2,4-D and 0.25 mg/L Kinetine.

Callus induction

Inside a laminar flow cabinet the axenic explant is introduced to a sterile Petri dish or flash and added with 20ml of MS media enriched with 2 .0 $mg.L^{-1}$ of 6 BAP , 0.1 $mg.L^{-1}$ NAA and 30 $g.L^{-1}$ sucrose, for adequate sterility a flame is passed over the opening of the recipients and they are sealed with aluminum paper with this, the incubation process will begin. Incubation specifications are: Maintain temperature at 25°C for 2 weeks with a day/night relation of 16/8hours. After this, a primary callus will be obtained. This callus will then be cut in four roughly equivalent portions and divided in four Petri dishes previously filled with the same MS media used for the primary callus. Each assay in their respective Petri dish will be added with 10mL of a Multi-walled carbon nanotubes-PBS (MWCNTs-PBS) each with a different concentration as shown in table 1 for a 4hr period at a temperature of 25°C.

Table 1: First experiment

Assay number	Concentration NTC-PBS (µg/ml)	Media exposure time (hrs.)
C-	0	4
1	1	4
2	2	4

3	4	4
4	6	4

Table 1: First experiment. Concentration and time parameters of four assays. A negative control will be added to this procedure.

Determination of fluorescence:

For the observation of the expected florescence in confocal microscopy, cells will be digregated by a mechanical method. The callus will be put in an Erlenmeyer flask (250ml) with 50m L of UM5 media and this will be maintained in agitation (150rpm, 25°C) until the callus is gone and a homogeneous solution is obtained. For the determination of total cells and transformed cells, a Neubauer chamber will be used for the counting of total cells. Then a confocal microscope will be used for the counting of fluorescent cells and a relationship of total/fluorescent will be adjusted. The preparation of the disgregate soluton is as follows: Glyceraldehyde at 50% concentration will be added at a Eppendorf vial (1ml) and with 800 µL of cell suspension solution. The Eppendorf will be covered with aluminum sheet and will be left to sit for 1min. When the time is over, 2 drops of the vial are added to a microscope slide and left in darkness until observation.

References:

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